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**14. ABSTRACT**

This project addresses the treatment of deleterious oxy-radical production following traumatic brain injury using anti-oxidant nanomaterials. Our evidence indicates that traumatic brain injury generates superoxide radical rapidly and persistently, which is exacerbated by systemic hypotension and resuscitation. Conventional anti-oxidant therapy is either not sufficiently potent nor long lasting. Nanomaterials synthesized in our collaborators in the Tour laboratory demonstrated potent anti-oxidant activity in preliminary results. We have developed several assays in the Kent laboratory that measure relevant anti-oxidant activity to our in-vivo purposes, including total anti-oxidant activity and activity against the superoxide radical, as well as testing for interactions with the fluorescent techniques planned for use in-vivo as well as assays to determine toxicity for the endothelial cell with which these materials

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Introduction: Oxidative stress is a prominent feature of TBI, in particular when accompanied by secondary insults such as hemorrhagic hypotension.[1] Antioxidant therapies have had limited success in treating TBI. We have established that one likely reason for this failure is that these agents are short acting and only minimally effective *in-vivo*. Initially, we identified carbon nanomaterials as potent antioxidants using a chemical ORAC assay. We hypothesize that these materials will be potent antioxidants *in vivo*, with a long duration of action. We are evaluating this hypothesis in two stages: evaluating the antioxidant strength of the materials in more biologically relevant *in vitro* assays and then we will pursue *in vivo* MTBI models.

Body of Report: ***SA #3.5.1:*** To determine if reduction in oxyradicals by BHT-modified PEGylated CNTs will improve vascular function following MTBI and improve neurological outcome following MTBI complicated by secondary insults.
***SA #3.5.2:*** To determine if antioxidant nanotubes are able to inhibit oxyradical induced endothelial activation and platelet aggregation.
***SA #3.5.3:*** To determine if modifying the PEG moieties will alter the distribution of antioxidant nanotubes into the brain itself following MTBI.

The work thus far has focused on *in vitro* work that has shown that our nanomaterials are suitable antioxidants to be used *in vivo* for further study in specific aims 3.5.1 and 3.5.3 and preliminary toxicity experiments as they relate to Specific Aim 3.5.2. At the outset of the grant, we had preliminary evidence that four carbon nanomaterials: pluronic wrapped single walled carbon nanotubes (p-SWCNT), PEGylated hydrophilic carbon clusters (PEG-HCC), butylated hydroxytoluene ionically bound to PEGylated hydrophilic carbon clusters (Ionic BHT/PEG-HCC), and BHT covalently bound to PEG-HCCs (Figure 1). Note that hydrophilic carbon clusters are produced from carbon nanotubes (CNTs) and at the time the grant was funded, these materials were identified as CNTs. However, we changed the name to better reflect their structure as the procedure cuts the CNTs into the size range of 30-60 nm (controllable length based upon the cutting temperature ranging from 23 to 70 °C), while destroying the sidewalls and leaving heavily carboxylated addends on these newly generated carbon clusters. One out of every four carbons bears an oxygen atom (by XPS), very unlike the nearly oxygen-free starting hydrophobic carbon material. These addends then render the carbon clusters water soluble and no longer soluble in typical organic solvents, hence they are termed HCCs.

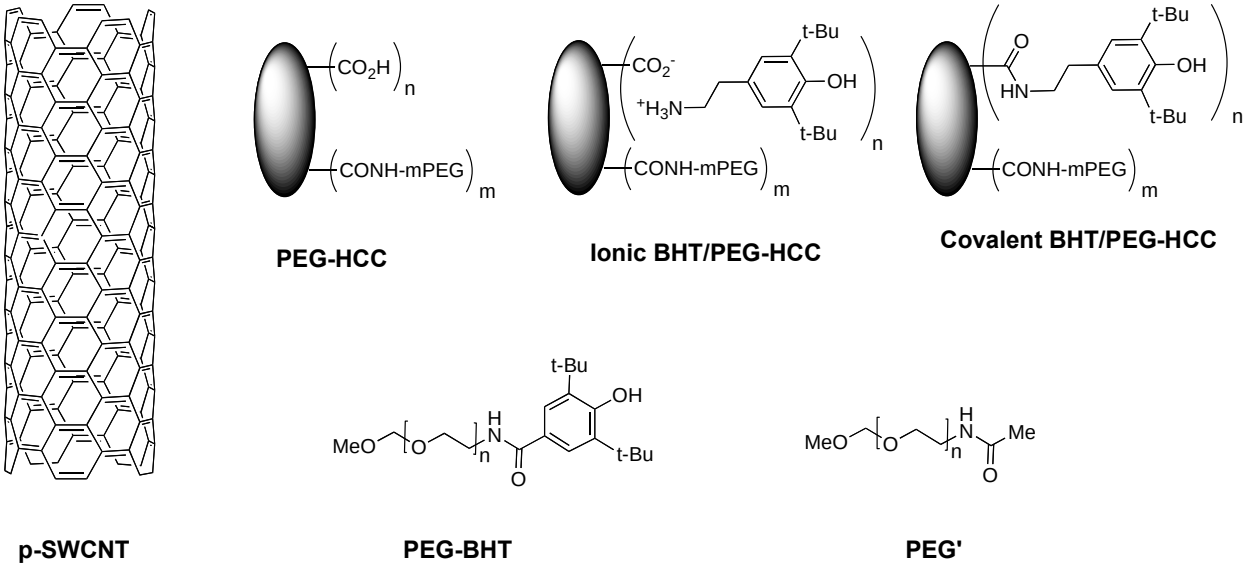



**Figure 1**. Carbon nanomaterials that are potent antioxidants and the appropriate controls.

We confirmed our preliminary results by performing an oxygen radical absorbency capacity (ORAC) assay using a chemical source for the oxygen radical (α,α′-azodiisobutyramidine dihydrochloride) (Table 1). We published a paper on this subject in the *Journal of the American Chemical Society*.[2] This paper is appended to this report. This assay identified the pluronic wrapped SWCNTs and ionic BHT/PEG-HCCs as the two most promising nanomaterials for *in vivo* work.

**Table 1.** Antioxidant strength of carbon nanomaterials determined with a chemically based ORAC assay.[a]

| Nanomaterial | Trolox Equivalents (TE) | Trolox Mass Equivalents (TME) |
|---|---|---|
| **p-SWCNT** | 14046 | 5.02 |
| **PEG-HCC** | 221 | 0.77 |
| **Ionic BHT/PEG-HCC** | 1240 | 4.31 |
| **Covalent BHT/PEG-HCC** | 532 | 1.85 |

[a]TE and TME values for HCC and SWCNT samples were adjusted to account for the contribution of the corresponding solubilizing groups PEG and Pluronic respectively, by subtracting the TE and TME values found for each solubilizing group. Error range is 15%.

Subsequently, we evaluated our nanomaterials using two additional antioxidant tests that make use of biologically relevant radicals, a ferryl myoglobin-based assay and a cytochrome *C*-based assay. The ferryl myoglobin assay is primarily a measure of activity against hydrogen peroxide radicals that lead to protein oxidation—an important form of damage during oxidative stress.

The cytochrome C assay assesses activity against the superoxide radical, which is of great interest given its early rise following TBI and potential for interaction with nitric oxide to produce the damaging effects of nitrotyrosolation if in high concentrations.

*Ferryl myoglobin assay*: This antioxidant assay is a colorimetric-based commercial kit (Sigma-Aldrich, CS0790) used to measure antioxidant activity against the ferryl myoglobin radical—a protein radical. All samples are compared to a standard curve from the soluble Vitamin E analog Trolox. The potency of each sample is expressed as Trolox mass equivalents (TME). Previously, nanomaterial antioxidant potentials were reported in both TEs (molar comparison) and TMEs (mass comparison). Due to the dramatic differences in molecular weight between the nanomaterial antioxidants and conventional antioxidants; henceforth, all data is given in TMEs. These units are more relevant for comparing the nanomaterial antioxidants with established doses of current antioxidants and planning animal dosing.

**Table 2.** Antioxidant strengths of nanomaterials and controls in ferryl myoglobin assay.

| Sample | Ferryl Myoglobin Assay (TME ± SD) |
|---|---|
| **p-SWCNT** | 123.8 ± 140.6 |
| **PEG-HCCs** | 3.3 ± 3.1 |
| **Ionic BHT/PEG-HCCs** | 17.3 ± 17.8 |
| **Covalent BHT/PEG-HCCs** | 4.4 ± 5.3 |
| **BHT-PEG** | 8.5 ± 8.9 |
| **PEG (Control)** | 20.2 ± 17.9 |

Although this assay was performed numerous times, we experienced great difficulty in eliminating 'background noise'. This can be seen in large errors associated with our TMEs and the fact that the negative control (PEG) had activity of 20.2 TME. We believe that the high variance is due to difficulty in preparing the nanomaterial samples such that their antioxidant behavior was in the range of the standard curve and the interference of PEG in the assay. We identified appropriate concentrations for the nanomaterials and we repeated this assay.

*Cytochrome c assay*: This antioxidant assay utilizes oxidized cytochrome *c* as a readout of superoxide levels produced by the enzyme and substrate xanthine oxidase and hypoxanthine, respectively. The level of oxidized cytochrome *c* is determined with and without antioxidants and plotted versus concentration.[3] The half maximal inhibitory concentration (IC50) in grams/liter (g/L) of the antioxidant can then be determined. Lower IC50 values indicate more potent antioxidants. One benefit of using this assay is that we have previously used the oxidation of cytochrome *c* as an *in vivo* monitoring technique for the detection of superoxide formation. Thus, demonstrating that the nanomaterials do not interfere with the assay will allow us to use this *in vivo* technique. Additionally, this assay robustly showed no antioxidant activity for our negative control, PEG' (Figure 2). The pluronic SWCNTs exhibited a very low IC50 (Figure 3).

The PEG-HCCs were active in this assay, but orders of magnitude less potent than the p-SWCNTs (Table 2). Unfortunately, we have not yet been able to obtain full IC50 curves for ionic BHT/PEG-HCC and covalent BHT/PEG-HCC. We have just prepared new batches of these samples in order to obtain this data.

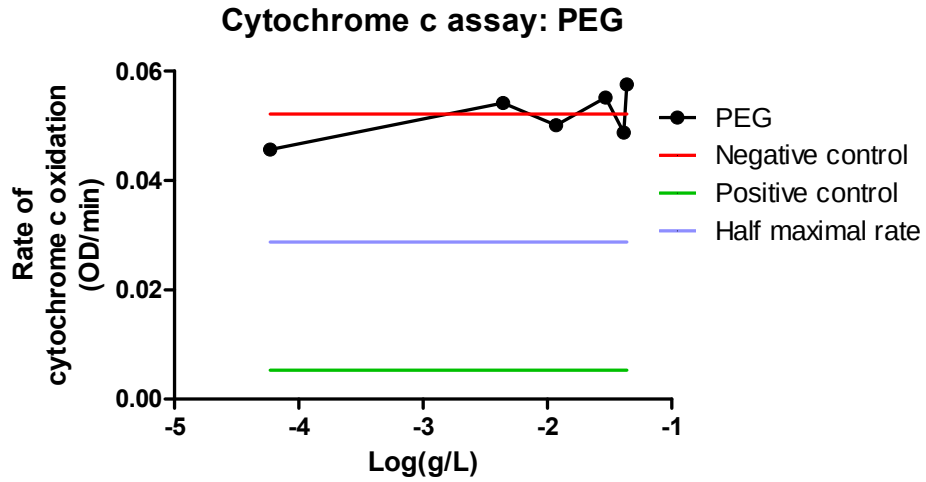



**Figure 2**: Graph of Polyethylene glycol (PEG) control from cytochrome *c* assay. The PEG has no antioxidant activity. Negative controls have no antioxidants. Positive controls contain excess amounts of superoxide dismutase (SOD).

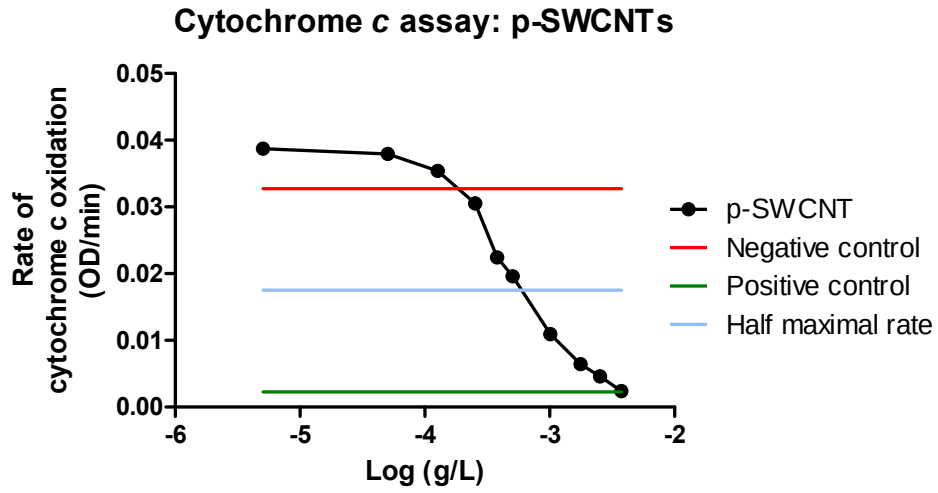



**Figure 3**: Graph of p-SWCNT from cytochrome *c* assay. The p-SWCNT has high antioxidant activity. Negative controls have no antioxidants. Positive controls contain excess amounts of superoxide dismutase (SOD). The half maximal rate line intersects the p-SWCNT line at the IC50 concentration (g/L).

**Table 3.** Antioxidant strengths of nanomaterials and controls in cytochrome *c* assay (IC50).

| Antioxidant Molecule | Cytochrome c Assay (IC50 ± SD) |
|---|---|

| | |
|---|---|
| **p-SWCNT** | 0.00153 ± 0.00188 |
| **PEG -HCCs** | 0.1706 ± 0.0585 |
| **Ionic BHT/PEG- HCCs** | NA* |
| **Covalent BHT-HCCs** | ∞ |
| **BHT-PEG** | ∞ |
| **PEG (control)** | ∞ |

* Concentration of antioxidant was not sufficient to obtain a full curve for IC50 calculation

∞ No concentration of nanomaterial or control exhibited activity against superoxide.

Dye interaction studies:

Future animal studies will include the use of fluorescent dyes to detect superoxide and nitric oxide. Nanomaterials have the potential to interfere with fluorescent signals, either through quenching or increasing fluorescence. To test our antioxidant compounds, we developed an *in vitro* assay for interaction of the nanomaterials with the fluorescent dye for nitric oxide (DAF2) which we use *in vivo* to determine the status of nitric oxide in the vasculature. We found conditions in which all four nanomaterials did not interfere with the DAF2 fluorescent dye for nitric oxide (Figures 4-6). We are currently optimizing an assay to test the interaction of the nanomaterials with the fluorescent dye for superoxide.

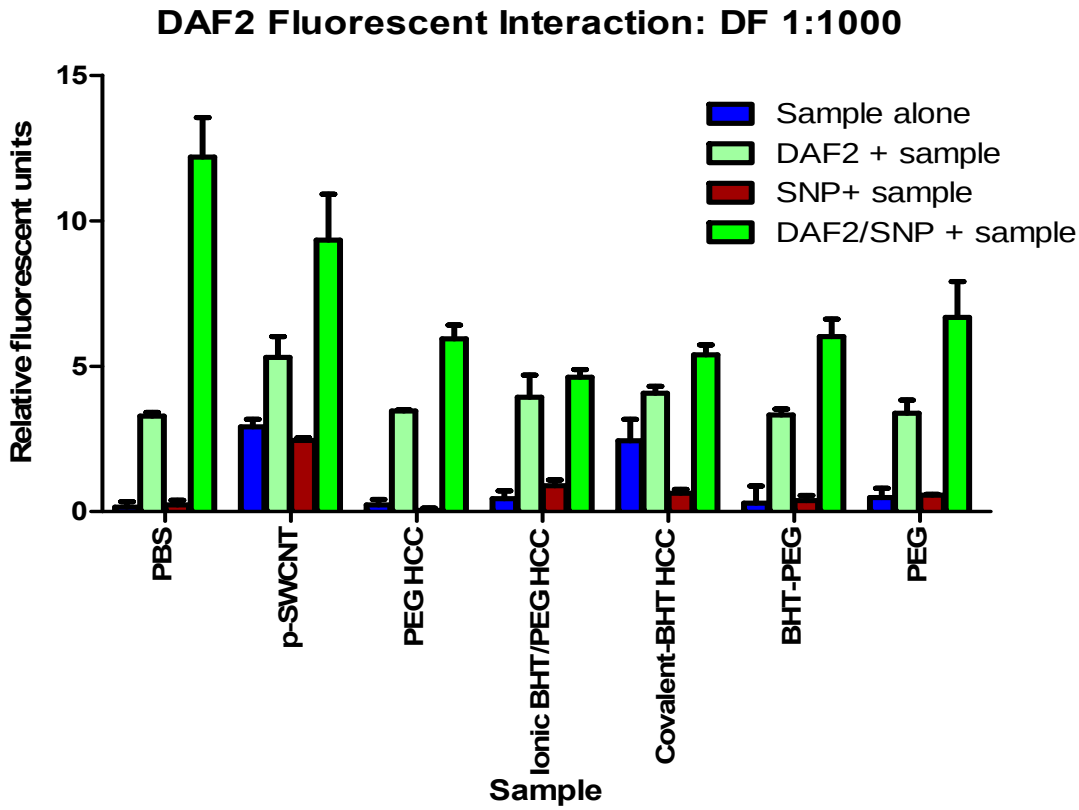



**Figure 6.** Interaction of nanomaterials and controls with fluorescent dye assay. Nanomaterials were diluted 1,000 times. This fluorescent assay is designed to detect nitric oxide. The fluorescent dye (DAF2) has its structure altered by reacting with nitric oxide and the product is more fluorescent than DAF2. Sodium nitroprusside (SNP) is the source of nitric oxide. The "sample" referred to in the legend indicates which compound (PBS or nanomaterial) was added to the reaction. Thus, the key is that the DAF2 + sample be significantly less fluorescent than DAF2/SNP + sample. At this dilution (1,000 fold), the nanomaterials and control compounds interfere with this discrimination. PBS=phosphate buffered saline.

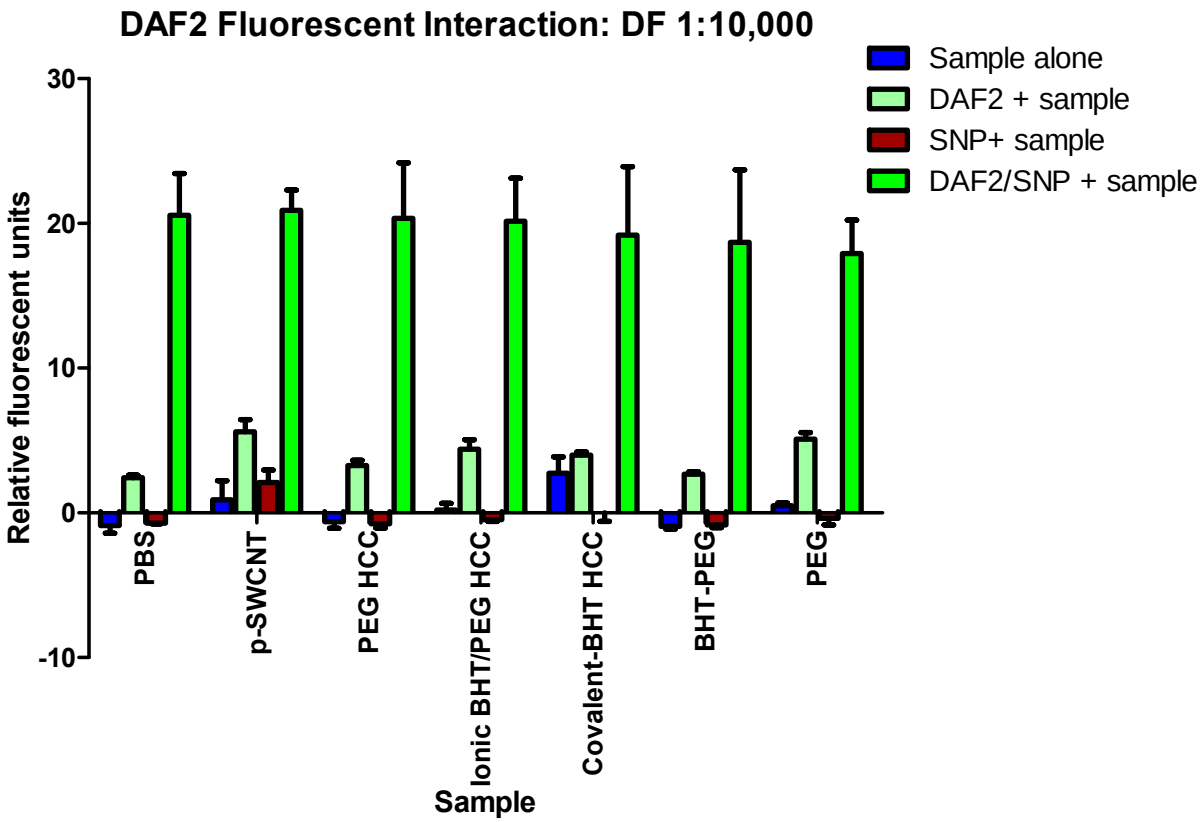



**Figure 7.** Interaction of nanomaterials and controls with fluorescent dye assay. Nanomaterials were diluted 1,000 times. This fluorescent assay is designed to detect nitric oxide. The fluorescent dye (DAF2) has its structure altered by reacting with nitric oxide and the product is more fluorescent than DAF2. Sodium nitroprusside (SNP) is the source of nitric oxide. The "sample" referred to in the legend indicates which compound (PBS or nanomaterial) was added to the reaction. Thus, the key is that the DAF2 + sample be significantly less fluorescent than DAF2/SNP + sample. At this dilution (10,000 fold), the nanomaterials and control compounds do not interfere with this discrimination.

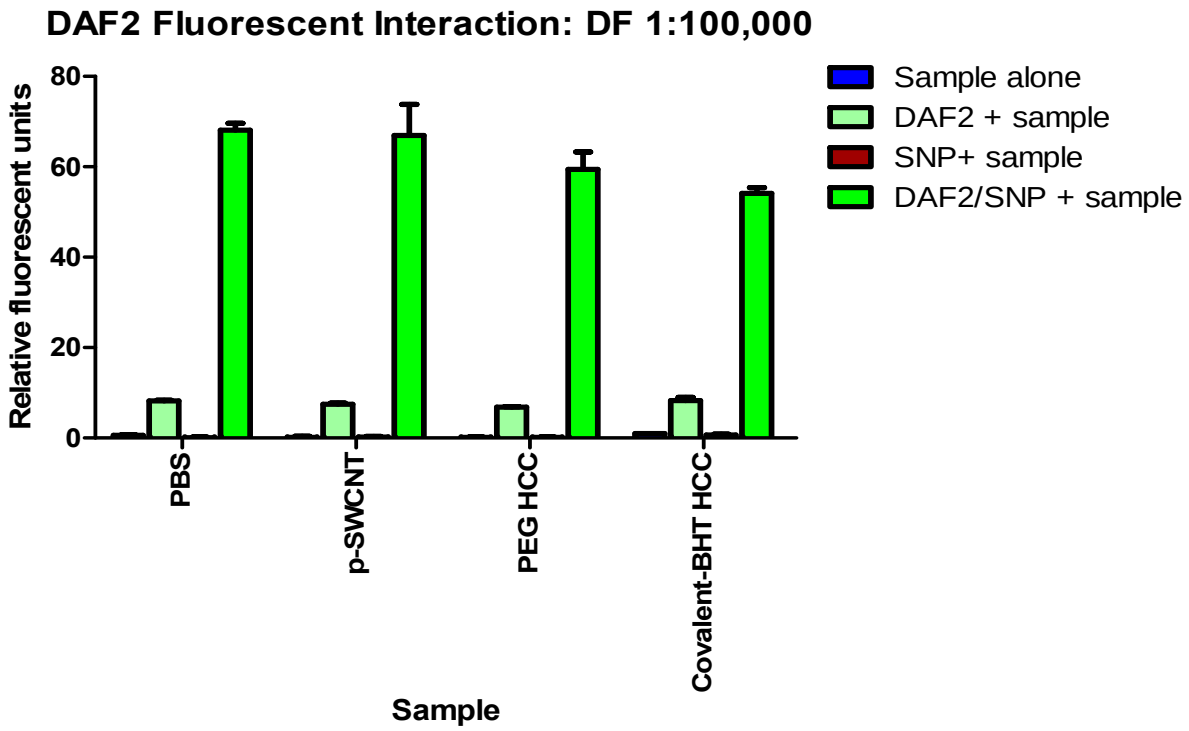



**Figure 8.** Interaction of nanomaterials and controls with fluorescent dye assay. Nanomaterials were diluted 1,000 times. This fluorescent assay is designed to detect nitric oxide. The fluorescent dye (DAF2) has its structure altered by reacting with nitric oxide and the product is more fluorescent than DAF2. Sodium nitroprusside (SNP) is the source of nitric oxide. The "sample" referred to in the legend indicates which compound (PBS or nanomaterial) was added to the reaction. Thus, the key is that the DAF2 + sample be significantly less fluorescent than DAF2/SNP + sample. At this dilution (100,000 fold), the nanomaterials and control compounds do not interfere with this discrimination.

*Distribution of nanomaterials:* We synthesized fluorescently labeled PEG-HCCs (FITC-PEG-HCC) for *in vivo* tracking (Aim 3.5.3). Bifunctional PEG polymers were prepared (Figure 9).

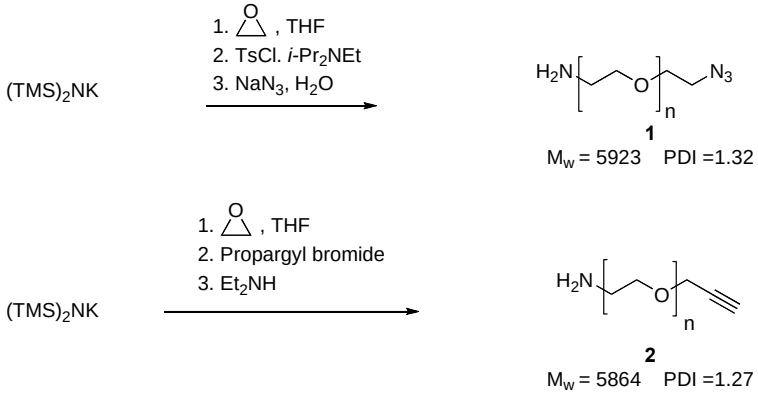



**Figure 9.** Synthesis of bifunctional PEGs for the preparation of labeled PEG-HCCs.

The bifunctional PEGs were then used to prepare FITC-PEG-HCCs (Figure 10). We are planning to use these labeled tubes for distribution studies in MTBI animal models.

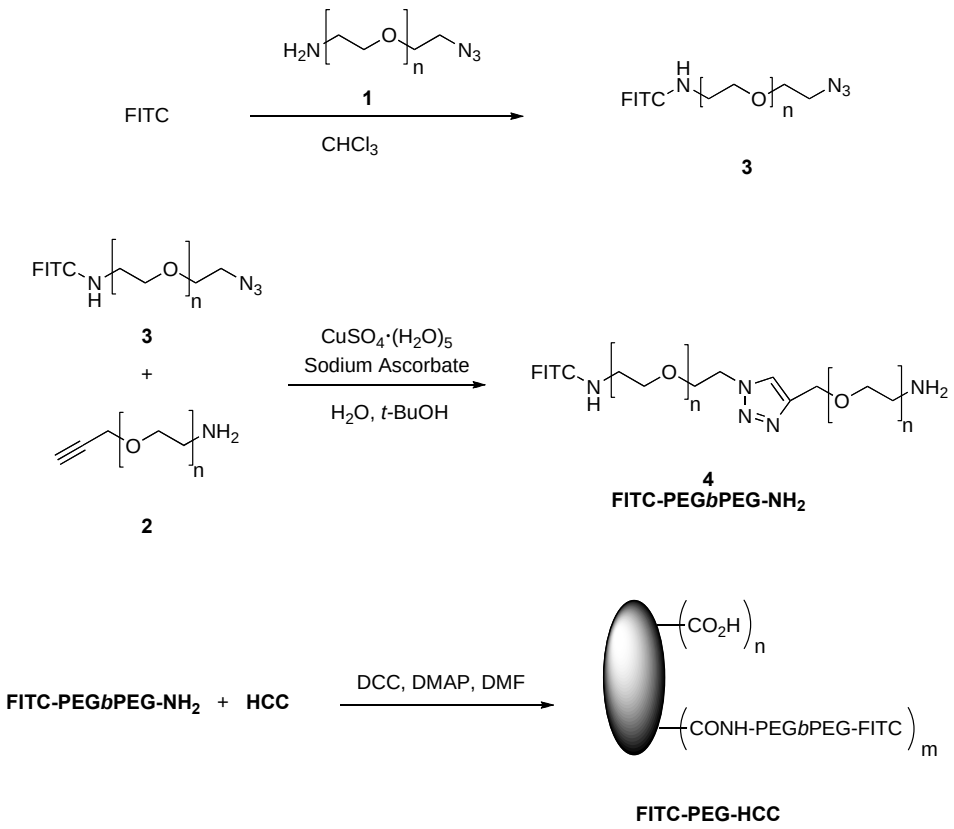



**Figure 10.** Synthesis of fluorescently labeled PEG-HCCs.

*Specific Aim 3.5.2: Toxicity of PEG-HCCs:*

Prior to beginning MTBI animal experiments, we wished to investigate the toxicity of our novel PEG-HCCs. Our strategy involves two types of assays, first are endothelial survival assays (trypan blue exclusion and WST-1 colorimetric assay) and a functional assay, the production of nitric oxide.

Cell survival: We initially used the standard fibroblast cell line NIH3T3 so that our results could be compared to others in the literature. We found that PEG-HCCs are non-toxic at their expected *in vivo* concentration ≤ 4 mg/L (Figure 4). We are currently exploring higher concentrations.

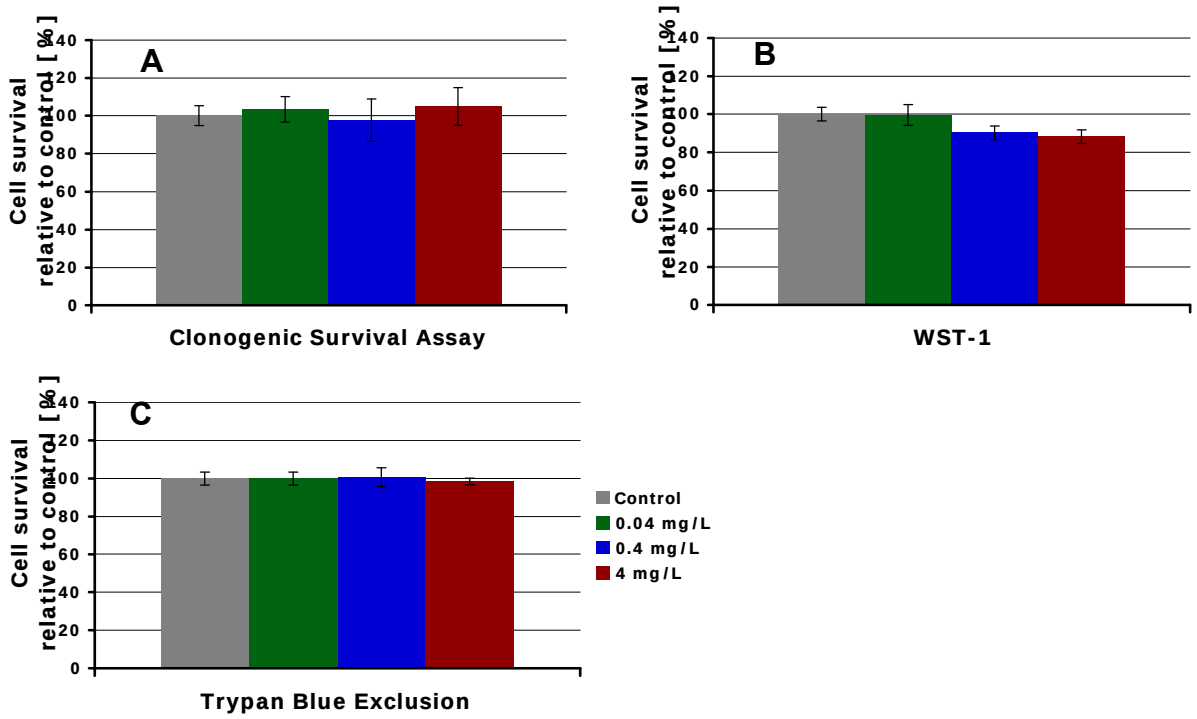



**Figure 4.** Three different viability assays (A-C) demonstrate that NIH-3T3 cells show no adverse effects when treated with PEG-HCCs.

We also examined the effect of PEG-HCCs on bEnd.3 cells, since these are a cell line derived from endothelial brain cells.

We first confirmed that the bEnd.3 cells expressed the endothelial marker, Von Willebrand Factor (VWF).

*bEnd.3 Cell cultures*

Mouse brain Endothelioma cells (bEnd.3) were obtained from American Type Culture Collection Manassas, VA. The cells were plated onto 25mm Poly-D-lysine coated round cover slips that were placed into 6 well culture plates. The cells are grown in DMEM containing 10% fetal bovine serum and kept in a $37^0$C 5% $CO^2$ incubator.

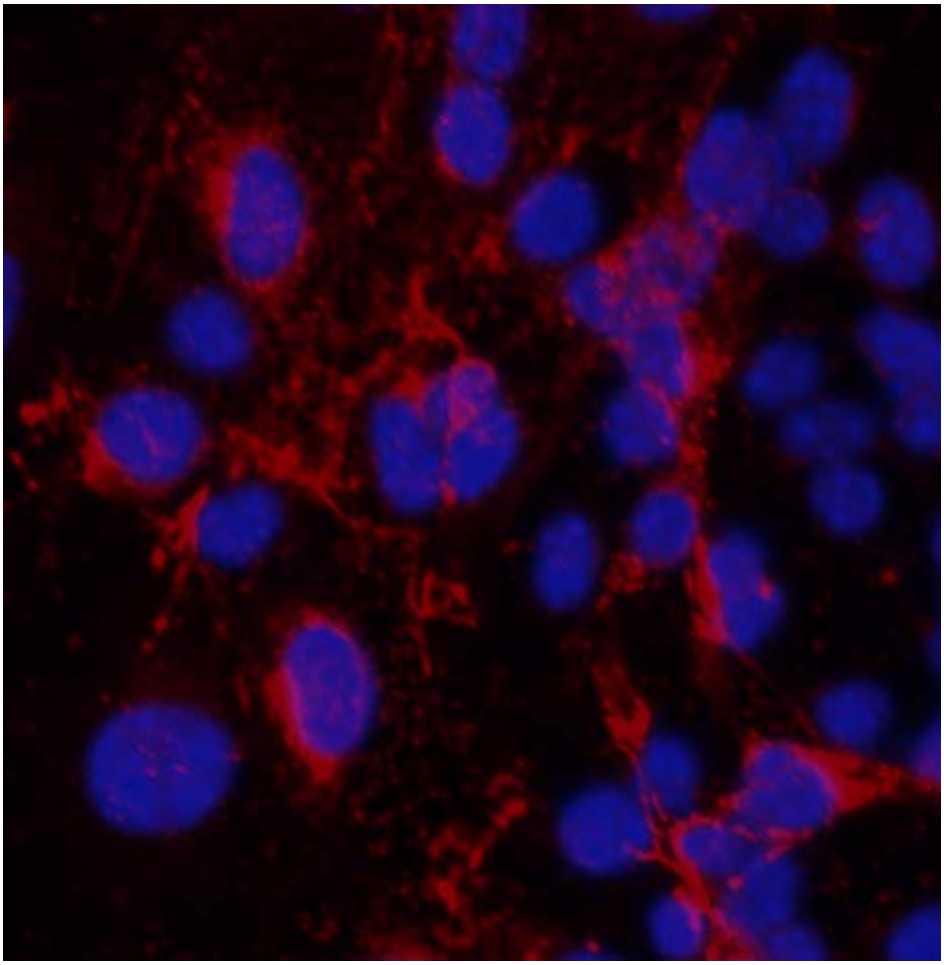
VWF Immunofluorescence experiment

To verify the identity of the cultured cells as vascular endothelial cell, immunofluorescence experiments for Von Willebrand Factor (VWF) were performed as follows:

Cells are plated at a density of 200,000 cells per 25mm cover slip and are grown to about 80% confluency. The cells are then washed 3 times with phosphate buffered saline (PBS) and fixed by the addition of $-20^0$C 100% methanol for 5 minutes. The cells are allowed to dry and then they are permeabilized by the addition of 0.1% Triton X-100/PBS for 30 minutes at room temp. The previous solution is aspirated and a blocking solution of 3% normal goat serum in 0.1%

Triton/PBS (NGST) is added for 30 min at RT. This solution is aspirated and a 1:400 dilution of the primary antibody (DAKO, polyclonal rabbit anti-human von Willebrand factor) in NGST is added. The cells are incubated at $4^0$C overnight. The cells are removed from $4^0$C and washed 3 times with PBS. The secondary antibody (Alexa fluor 594 goat anti-rabbit IgG) in NGST is added at a dilution of 1:1000. The cells are incubated shaking at RT for 4 hours in the dark. After 4 hours the cells are washed 3 times with PBS and then the cover slip is removed from the well and attached to a microscope slide using a clear adhesive. The cover slip is then covered with another using Vectastain mounting medium containing DAPI. The slide is allowed to dry in the dark at room temperature. The dried slides are then visualized using a Nikon Eclipse 80i epi-fluorescense microscope and Metamorph software. The cell nuclei fluoresce blue and the VWF red (see figure below).

We verified that these cells express VWF as illustrated in the following image:



This figure illustrates the identification of cultured cells as endothelium based on expression of VWF (red).

Toxicity assay: We performed these assays at higher concentrations based on our results with NIH3T3 cells. We have only performed each assay once with the b.End3 cells and conflicting results were obtained. The trypan blue exclusion assay indicated that there was no toxicity for concentrations up to 200 mg/L, while the WST-1 assay showed profound toxicity for 100 mg/L. The discrepancy could be due to either the different time points for the two assays (6 hrs for TB vs. 24 hrs for WST-1) or the nanomaterials interfering with the colorimetric assays. We are currently exploring these possibilities.

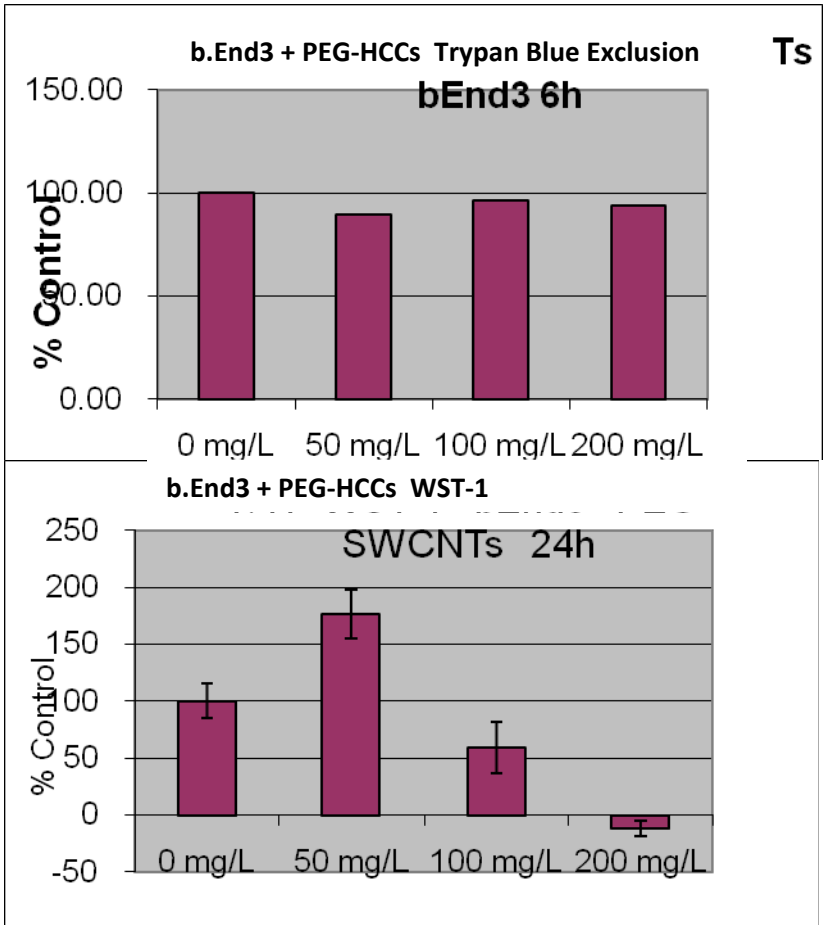


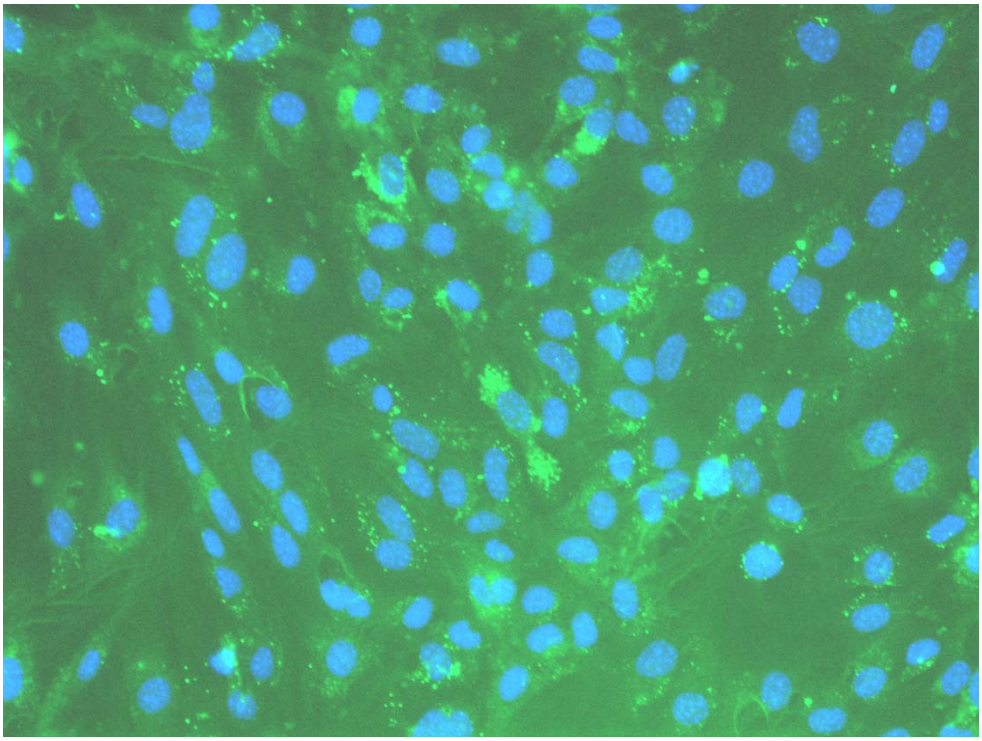

**Figure 5.** Trypan blue exclusion and WST-1 assay for bEnd.3 cells treated with PEG-HCCs.

Functional Assay: DAF-2 Immunofluorescence experiment

A second phase of in-vitro toxicity experiments involves the functional ability of endothelial cells to produce nitric oxide both basally and after stimulation. Our initial experiments were intended to demonstrate that the culture conditions described above resulted in functionally active endothelial cells as manifested by the ability to express nitric oxide. The same principle as applied in Specific Aim 3.5.1 and eventually in-vivo is to use the activation of fluorescence through species specific dyes, in this case DAF-2, to detect nitric oxide. The methods used are described below:

A 1:1000 dilution of a 5uM solution of DAF-2 **(**4,5-Diaminofluorescein.diacetate) was added to bEnd.3 cells grown on cover slips in 6 well plates. The cells were incubated for 5 minutes at $37^0$C 5% $CO^2$. The media was aspirated and the cells washed 3 times with PBS and fixed with -$20^0$C methanol for 5 minutes. The cover slips were removed from the wells and allowed to dry. The cover slips were mounted onto microscope slides and covered with another using Vectastain mounting medium containing DAPI. The slides were analyzed using the Metamorph software. The cell nuclei fluoresce blue and the DAF-2 green.

The ability to detect nitric oxide is illustrated in the following figure:



This figure illustrates our ability to generate viable endothelial cell cultures as a platform in which to test any functional deterioration with candidate nanotubes.

Our next step will be to establish an *in vitro* model for assessing superoxide production by bEnd.3 cells and then to test for toxicity of our candidate nanomaterials for functional toxicity based on impairment of nitric oxide release or production of superoxide. We will also perform immunohistochemical studies on expression of endothelial activation markers as well as whether the endothelium is stimulated to bind platelets, both of which would indicate potential toxic effect of the agents.

*Outlook*: While additional *in vitro* experiments remain, our preliminary results are promising and suggest that the nanomaterials are effective antioxidants for biologically relevant radicals and that the nanomaterials are non-toxic at biologically relevant concentrations.

Key Research Accomplishments:

- Prepared antioxidant nanomaterials on large scale
- Measured the potency of these nanomaterials using an ORAC assay; the nanomaterials are stronger than most conventional antioxidants
- Determined that the nanomaterials are antioxidants for superoxide using assays developed in our laboratories
- Developed *in vitro* biological assays with cultured NIH3T3 and bEnd.3 cells
- Determined that *in vitro* with NIH3T3 cells, the nanomaterials are non-toxic at biologically relevant concentrations
- Determined that, at biologically relevant concentrations, the nanomaterials do not interfere with our proposed *in vivo* assay for antioxidant strength (fluorescence probes)
- Developed a modular synthetic route to labeled PEG-HCCs. Used this synthetic route to prepare fluorescently labeled PEG-HCCs.

Reportable Outcomes:

- *JACS* paper (Lucente-Schultz, 2009)
- Lucente-Schultz obtained a Masters, Rice University
- Applied for an NIH Grant (RFA-OD-09-003), "Radical Scavenging Nanotubes as an AD Treatment"

Conclusion: Between the Kent and Tour laboratories, in this first year, a great deal of training and research was accomplished. A postdoc and a graduate student were trained in the synthesis and characterization of the nanomaterials required for this project. Another graduate student was trained in the *in vitro* techniques necessary for this project. With these skilled researchers, we were able to determine that our nanomaterials are potent antioxidants. We published a paper on this phenomenon, using a chemical radical source. We have gone on to, preliminarily, demonstrate that the nanomaterials are antioxidants for superoxide, a biologically relevant radical. We have also shown *in vitro* that at biologically relevant concentrations, the nanomaterials are non-toxic and do not interfere with our *in vivo* assay. As we are narrowing down the choices of nanomaterials, we are in the process of obtaining the necessary animal protocols and are looking forward to carry out *in vivo* studies using these antioxidant nanomaterials to treat MTBI.

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